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(54) Title: TRANSGENIC PLANTS USING THE TDC GENE FOR CROP IMPROVEMENT

(57) Abstract

The present invention provides nucleic acid constructs and methods of producing transgenic plants having improved resistance to fungi, bacteria, and/or nematodes, wherein the enhanced resistance arises from enhanced expression of a tryptophan decarboxylase (TDC) gene construct.

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TRANSGENIC PLANTS USING THE TDC GENE FOR CROP IMPROVEMENT

This application claims the benefit of U.S. Provision Application No. 60/054,316, filed July 31, 1997.

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to transgenic plants having surprisingly improved fungal, bacterial, and/or nematode disease resistance, wherein the enhanced resistance arises from enhanced expression of a tryptophan decarboxylase (TDC) gene.

Summary of the Related Art

Crop losses incurred by diseases and pests have a major negative economic impact upon all sectors of agriculture today. Despite attempts to control these losses through various crop husbandry techniques (*e.g.*, crop rotation), breeding of resistant cultivars and application of agrochemicals, losses remain unacceptably high. As of 1987, approximately 37% of all crops produced worldwide are lost to pests such as insects (13%), diseases (12%), and weeds and grasses (12%). Dependence on agrochemicals is not only expensive, but it is also detrimental to the environment and generally regarded as unhealthy for living creatures. Over the last decade, agricultural biotechnology has provided some solutions for disease and insect management. The most intensive global efforts in agricultural biotechnology today are directed toward the design and implementation of effective transgene-mediated disease and pest resistance strategies.

The tremendous losses incurred by pathogenic fungi on crops worldwide has fueled the interest to develop rational and effective anti-fungal strategies in transgenic plants (for review, see Cornelissen and Melchers (1993) *Plant Physiol.* **101**, 709-712). One of the earliest concepts, and to date, one of the most successful anti-fungal strategies has been to utilize fungal cell wall-degrading enzymes to attack an invading pathogen. Proteins that possess chitinase or β-glucanase activity have been purified and characterized from numerous plant, fungal, and bacterial sources (Kauffman *et al.* (1987) *EMBO J.* **6**, 3209-3212; Legrand *et al.* (1987) *Proc. Natl. Acad. Sci. USA* **84**, 6750-6754). The genes that encode for some of these hydrolytic enzymes have been cloned, sequenced, and subsequently overexpressed in transgenic plants. The single, unifying conclusion that has emerged from these numerous studies is that while modest levels of fungal protection can

be afforded by overexpression of these enzymes alone, significantly enhanced protection can often be achieved by co-expression of chitinase and β-glucanase together. To date, encouraging results have been reported in transgenic tomato against *Fusarium solani* (Jongedijk *et al.* (1995) *Euphytica* **85**, 1-8) and in transgenic tobacco against *Rhizoctonia solani* (Jach *et al.* (1995) *Plant J.* **8**, 97-109). Extensive field testing remains necessary, however, to determine whether these resistance levels are sufficient to confer commercially-significant phenotypes.

An alternative anti-fungal technology that has slowly been gaining attention involves overexpression of small peptides with antimicrobial activity in transgenic plants. Some of these peptides, isolated from non-plant sources such as frog (magainins) and moths (cecropins), form amphipathic α-helices upon association with microbial plasma membranes, insert into the membranes and interact with one another to initiate pore formation, causing cell leakage and eventual cell death (for review, see Boman, H.G. (1991) *Cell* **65**, 205-207). The expression of cecropins in transgenic tobacco, however, has not led to any significant levels of disease resistance (Florack *et al.* (1995) *Transgenic Res.* **4**, 132-141). Other antimicrobial peptides, termed defensins (for review, see Broekaert *et al.* (1995) *Plant Physiol.* **108**, 1353-1358) have been isolated from radish (Terras *et al.* (1992) *J. Biol. Chem.* **267**, 15301-15309) and barley (Mendenez *et al.* (1990) *Eur. J. Biochem.* **194**, 533-539) and feature a more complex three-dimensional structure that includes cysteine-stabilized triple anti-parallel β sheets together with an α-helix. Terras *et al.* (1995) *Plant Cell* **7**, 573-588, reported very good levels of protection against infection by *Alternaria* in transgenic tobacco that overexpressed the radish AFP2 protein. A threshold level of AFP2 peptide (which was not easily obtained) in the transgenic plants was required to detect any significant level of disease resistance, however.

Other anti-fungal proteins include ribosome-inactivating proteins (RIP's), which act by inhibiting protein synthesis in target cells by a modification of the 28S rRNA. RIP's do not affect the ribosomes of the plants in which they are produced, but can be effective against fungal ribosomes. A barley RIP under control of a wound-inducible promoter was reported to show increased resistance to *Rhizoctonia solani* (Logemann *et al.* (1992) *Biotech* **10**, 305-308). Finally, Alexander *et al.* (1993) *Proc.Natl. Acad. USA* **90**, 7327-7331, demonstrated that constitutive high-level expression of tobacco PR-1a (pathogenesis-related) protein (of unknown activity) in transgenic tobacco resulted in increased resistance to *Phytophthora parasitica and Peronospora tabacina.*

Manipulation of the plant's own defense system -- systemic acquired resistance (SAR) -- has drawn tremendous attention in recent years. Activation of this complex network of defense-related genes gives rise to protection against a wide range of pathogens, including bacteria, fungi, and viruses. Attempts to utilize the entire complex of defense-related genes in the SAR repertoire include the two-component system, which has been described by de Wit, P.J.G.M. (1992) *Annul Rev. Phytopathol.* **30**, 391-418, the application of chemical inducers of SAR like 2,6-dichloroisonicotinic acid (Kessmann *et al.* (1994) *Annul Rev. Phytopathol.* **32**, 439-459), and the constitutive expression of proposed intermediates (*e.g.*, active oxygen species like hydrogen peroxide) in the signal transduction pathway for SAR that effectively activates the SAR response (Wu *et al.* (1995) *Plant Cell* **7**, 1357-1368). These approaches hold tremendous potential for conferring broad spectrum disease resistance, but with the exception of chemical inducers of SAR, are in the development phase and remain largely unproven.

Plants produce a rich diversity of secondary metabolites, which do not seem necessary for their basic metabolism, but appear to contribute to their environmental fitness and adaptability. These secondary compounds are responsible for aroma (monoterpene indole alkaloids) and color (anthocyanins and carotenoids) and are commercial sources of numerous important pharmaceutical (alkaloids) and industrial chemicals. Their importance has encouraged intensive investigation of the regulation and control of the biosynthetic pathways, and more recently, how these pathways can be manipulated through "metabolic engineering," first coined by Bailey, J.E. (1991) *Science* **251**, 1668-1675, as "the improvement of cellular activities by manipulation of enzymatic, transport, and regulatory functions of the cell with the use of recombinant DNA technology."

The enzymology associated with the biosynthesis of alkaloids has been the subject of much study. The tropical plant, *Catharanthus roseus* (periwinkle), forms a wide range of terpenoid indole alkaloids (TIA's), some of which have important medicinal applications. The leaf-derived alkaloids, vinblastine and vincristine, and the root-derived alkaloids, ajmalicine and serpentine, are valuable drugs for treatment of cardiac/circulatory diseases and tumors, respectively. It is generally accepted that protoalkaloid production is the first committed step in the TIA pathway of *Catharanthus*. Consequently, tryptophan decarboxylase (TDC), the enzyme that catalyzes this bridge reaction between primary (amino acid) and secondary (alkaloid) metabolic pathways, has drawn much attention. TDC catalyzes the decarboxylation and conversion of L-tryptophan into tryptamine.

Tryptamine and secologanin, another secondary compound, are then condensed to form strictosidine, the precursor for all TIA's in *Catharanthus*.

For microbes and plants alike, the rate-limiting enzyme for tryptophan biosynthesis has been identified to be anthranilate synthase (AS), a heterodimeric enzyme composed of an α and β subunit, which catalyzes the conversion of chorismate to anthranilate. Tryptophan has been demonstrated to be a negative feedback regulator of the microbial enzyme. In microbes, when tryptophan levels are sufficiently high, tryptophan binds to an allosteric site on the α subunit, thereby inactivating the enzyme. Although similar observations have not been made for the plant enzyme, other studies suggest that AS is the rate-limiting enzyme for tryptophan biosynthesis. Plant mutants characterized by elevated tryptophan levels (~3-fold) have been found to contain amino acid changes in their anthranilate synthase coding regions.

More recently, biochemical studies with the two purified anthranilate synthase α subunits (ASα1 and ASα2) from *Ruta graveolens*, and molecular analysis of the genes that encode these proteins have provided some new insight into the complexities of tryptophan biosynthesis. *R. graveolens*, like *C. roseus*, is a medicinal plant which produces a large number of tryptophan-derived monoterpenoid indole alkaloids. Bohlmann *et al* (1996) *Plant Physiol.* **111**, 507-514, separately purified the ASα1 and ASα2 subunits from *E. coli* strains designed to overexpress the two proteins. They noted that while the ASα2 enzyme was completely feedback inhibited by ~10 mM tryptophan, the activity of the ASα1 enzyme was essentially unaffected by tryptophan levels approaching 100 mM. They further demonstrated that ASα1 (but not ASα2) transcript levels and enzyme activity were dramatically induced in *Ruta* cultures that had been exposed to fungal elicitors. They concluded that ASα1 and ASα2 participate equally in the housekeeping role of maintaining appropriate tryptophan levels. However, upon exposure to fungal elicitors that induce the TIA pathway, the tryptophan feedback-resistant ASα1 becomes responsible for supplying the copious amounts of tryptophan that will be required for conversion into tryptamine and ultimately, indole alkaloids. Thus, the tryptophan-resistant ASα1 becomes a specific enzyme for secondary metabolism.

In 1988, De Luca *et al.* (1988) *Plant Physiol.* **86**, 447-450, noted that TDC enzymatic activity was highly regulated in germinating seedlings, supporting similar observations made in cell suspension cultures. Later, De Luca *et al.* (1989) *Proc. Natl. Acad. USA* **86**, 2582-2586, described

the cloning and characterization of a TDC cDNA clone from *Catharanthus* seedlings. The availability of the TDC gene as a nucleic acid probe has helped to elaborate the developmental and environmental cues that affect TDC expression during plant growth and in cell suspensions. For example, Pasquali *et al.* (1992) *Plant Mol. Biol.* **18**, 1121-1131, reported that TDC steady-state transcript levels were most abundant in roots, moderately abundant in leaves and barely detectable in the flowers and stems of 3-month-old *Catharanthus* plants. They further demonstrated that TDC expression in cell suspension cultures was down-regulated by addition of auxin, but strongly induced by treatment with fungal elicitors. This report, along with others (Roewer *et al.* (1992) *Plant Cell Rep.* **11**, 86-89; Berlin *et al.* (1993) *Transgenic Res.* **2**, 336-3444; Goddijn *et al.* (1992) *Plant Mol. Biol.* **18**, 1113-1120; and Goddijn *et al.* (1995) *Transgenic Res.* **4**, 315-323), helped establish the importance of TDC as the first committed step in TIA biosynthesis.

Given its pivotal role in TIA biosynthesis in plants, efforts were initiated to overexpress TDC in transgenic plants for the purpose of increasing tryptamine levels. Songstad *et al.* (1990) *Plant Physiol.* **94**, 1410-1413, placed the TDC cDNA under control of the strong, constitutive cauliflower mosaic virus (CaMV) 35S RNA promoter and introduced this transgene into tobacco. Transgenic tobacco lines were recovered which accumulated up to 250 times their normal levels of tryptamine (in excess of 1 mg/gram fresh weight [gfw]) with no apparent deleterious effect on plant growth and development.

In contrast to tobacco, transgenic canola, which overexpressed the same TDC transgene, exhibited a very striking phenotype. Chavadej *et al.* (1994) *Proc. Natl. Acad. USA* **91**, 2166-2170, reported that their best transgenic canola line never expressed >9% of the TDC-specific activity and only accumulated 2% of the tryptamine found in the most active transgenic tobacco line. Despite the relatively low TDC activity and tryptamine levels, the pool of available soluble tryptophan for tryptophan-derived indole glucosinolate production was so depleted that glucosinolate levels were reduced in all tissues, especially in mature seeds where glucosinolates accumulated to only 3% of that which were found in non-transformed seeds. Thus, TDC effectively out-competed the indole glucosinolate biosynthetic enzymes for available tryptophan by diverting it to the production of tryptamine.

In TDC-expressing potato, tryptamine accumulation was found to be very tissue specific (Yao *et al.* (1995) *Plant Cell* **7**, 1787- 1799). While tryptamine accumulated to high levels in the leaves of transgenic potato plants, tryptamine was undetectable in the tubers and was only detected

after wounding or fungal elicitor treatment. The re-direction of tryptophan into tryptamine resulted in a dramatic decrease in the levels of soluble tryptophan, phenylalanine, and phenylalanine derived phenolic compounds, including chlorogenic acid, the major soluble phenolic ester in potato tubers. This 2-3-fold reduction in phenolic esters led to reduced synthesis of polyphenolic compounds like lignin because of a limited supply of phenolic monomers. This in turn led to altered cell wall structures in the tubers, which proved to increase susceptibility of the tubers to infection by *Phytophthora infestans*.

Thomas *et al.* (1995) *Plant Physiol.* **109**, 717-720, extended the observations made by Songstad and co-workers in TDC-expressing tobacco when they reported that tryptamine-accumulating tobacco plants adversely affected whitefly development and reproduction. *Bemisia tabaci*, the sweet potato whitefly, was used to test the effects of tryptamine on insect feeding and development. Whitefly emergence tests revealed that pupae emergence (to adulthood) on TDC-expressing plants was typically reduced 3 to 7-fold relative to control plants. They speculated that tryptamine may exert its anti-whitefly effect(s) during either larval and pupal development and/or adult selection of a leaf for feeding and oviposition.

Pasquali *et al.*, *supra*, and Roewer *et al.* (1992) *Plant Cell Rep.* **11**, 86-89, noted that enzymes in the *Catharanthus* TIA biosynthetic pathway, including TDC, were coordinately induced upon exposure to fungal elicitors. A short report by Miyagawa *et al* (1994) *Biosci. Biotech. Biochem.* **58**, 1723-1724, detected the presence of fluorescent compounds deposited at the site of infection by powdery mildew in barley. One of these compounds, tryptamine, was suggested to be acting as an anti-fungal agent.

However, this collection of observations answered neither the question of whether tryptamine, normally only a precursor in the TIA biosynthetic pathway, could itself contain broad-spectrum anti-fungal activity, nor what levels could be attained *in planta* to be sufficient to confer enhanced resistance to infection by fungal pathogens. To date, the answers to these questions have remained elusive.

SUMMARY OF THE INVENTION

We have surprisingly discovered that tryptamine can effectively inhibit *in vitro* and *in vivo* growth of both phytopathogenic bacteria and fungi. Furthermore, tryptamine is shown herein to inhibit phytopathogenic nematodes. As a result, the present invention affords constitutive expression

in plants of the gene encoding tryptophan decarboxylase (TDC), the enzyme that converts tryptophan to tryptamine, to confer enhanced resistance to infection to a broad spectrum of phytopathogenic fungi, bacteria, and nematodes. Similarly the AS gene can boost this pathway, making more tyrptamine.

The present invention further provides a gene construct containing a promoter and a DNA sequence encoding a protein with tryptophan decarboxylase activity and/or AS activity.

The invention also provides transgenic plants and multicellular plant tissue having enhanced fungal and/or bacterial disease resistance and/or nematode resistance, wherein the enhanced resistance is a result of expression of a TDC and/or AS transgene.

The foregoing merely summarizes certain aspects of the invention and are not intended, nor should they be construed, as limiting the invention in any manner. All patents, patent applications, and publications are hereby incorporated by reference in their entirety.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. *Chimeric (A)* E35S::TDC::nos *and (B)* UBQ3::TDC::nos *constructs.* A DNA fragment containing the duplicated enhancer region, promoter, transcription initiation site and 5′ untranslated region (UTR) from the CaMV 35S RNA region was fused to the 5′ UTR of the TDC cDNA from plasmid pTDC5. A second DNA fragment containing the promoter, transcription initiation site and 5′ UTR from the *Arabidopsis thaliana UBQ3* gene was fused to the 5′UTR of the TDC cDNA from plasmid pTDC5. A DNA fragment containing the polyA addition signal from the nopaline synthase gene *(nos) is* responsible for transcript maturation at the 3′ end in both transgenes.

Figure 2. Flowers from tryptamine-accumulating petunias show enhanced resistance to infection by *Botrytis cinerea.* Newly opened flowers from greenhouse petunias (3 flowers/line) were detached, placed in water and then inoculated with a freshly prepared *Botrytis* spore suspension (in 0.005% Tween 20 to discourage spore clumping) containing 0.25×10^3 or 1×10^3 spores/ml. After the flowers were sprayed to the point of runoff with an atomizer, the inoculated flowers were placed in a humidity chamber and incubated at room temperature for 4 days. Disease progression was monitored daily (up to 97 h) using a disease rating scale ranging from 0 to 5 where 0 = uninfected; 1 = 1-15 small, necrotic lesions; 2 = 15-30 larger, necrotic lesions; 3 = lesions begin to coalesce; 4 = lesions almost fully coalesced; and 5 collapsed flower.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present inventors have surprisingly discovered (as is demonstrated in the Examples, *infra*) that enhanced TDC expression can confer on plants resistance to a broad spectrum of both bacterial, fungal, and nematode phytopathogens. In accordance with this discovery, the present invention comprises methods and nucleic acid constructs for enhancing resistance in plants to phytopathogenic bacteria, fungi, and nematodes by transforming plant cells with the gene coding for tryptophan decarboxylase (TDC). Although the invention is not limited by any theory of action, we believe that enhanced TDC expression results in enhanced phytopathogenic resistance through increased levels of tryptamine and/or alkaloids produced therefrom. The invention further comprises transgenic plant tissue thereby produced.

In a first aspect, the invention provides nucleic acid constructs comprising the TDC gene. Any plant TDC gene can be used. Upon transformation of plant cells, these constructs are useful for conferring enhanced fungal, bacterial, and/or nematode resistance to a wide variety of plants and against a broad spectrum of phytopathogenic fungi, bacteria, and nematodes.

In another embodiment of this aspect, the invention provides nucleic acid constructs comprising an AS (preferably ASα1) and a TDC transgene. Any plant AS gene can be used. Conferral of resistance to bacterial, fungal and nematode phytopathogens by overexpression of tryptamine (and/or alkaloids produced therefrom) can be enhanced by ensuring a constant, sufficient supply of its precursor, tryptophan. Published observations on the anthranilate synthase α subunits (ASα1 and ASα2) indicate that constitutive expression of the *Ruta* ASα1 subunit in heterologous plants can circumvent the normal regulatory mechanisms in transformed cells and lead to elevated tryptophan levels. We have found that co-transformation with ASα1 and ASα2 led to enhanced levels of tryptophan and an increased amount of tryptamine. Co-introduction of constitutively-expressed AS (preferably ASα1) and TDC transgenes should provide increased tryptophan levels for immediate conversion into tryptamine. A single plasmid harboring both constitutively-expressed transgenes can be viewed as portable expression cassette for increasing tryptamine levels in virtually any plant. Alternatively, an AS transgene could be used by itself.

In plants, tryptophan biosynthesis is believed to reside exclusively in the plastid; no cytosolic localization is implicated. This conclusion has been solidified by the observation that all the genes in the tryptophan biosynthesis pathway that have been cloned to date encode proteins that contain a recognizable transit peptide at their N-terminus to direct the proteins to the organelle. No gene has

thus far been cloned which lacks this feature, thus bolstering the argument that tryptophan biosynthesis is exclusively plastid-localized. Therefore, expression of the TDC gene in the plastid will result in better substrate availability (as well as higher TDC activity as is generally associated with plastid gene expression), which should lead to higher levels of tryptamine accumulation.

Accordingly, the nucleic acid constructs according to this aspect of the invention will further comprise targeting regions at the 3′ and 5′ ends, which regions target the constructs according to the invention to the plant nucleus or plastid.

In this aspect of the invention, any plant promoter can be operatively linked to the TDC and/or ASα1 genes. In a preferred embodiment, the constructs according to this aspect of the invention will preferably be operatively linked to the UBQ3, UBQ10, CaMV 35S RNA, or the enhanced version of the CaMV 35S RNA (E35S) promoter. In another preferred embodiment, the constructs further comprise a pUC-based vector containing the 3′ flanking region of the nopaline synthase gene (*nos*) from *Agrobacterium tumefaciens*. The promoters of the *UBQ3* and *UBQ10* genes, members of the polyubiquitin gene family in *Arabidopsis thaliana*, have been described by Norris *et al.*, *Plant Mol. Biol.* 21:8995-8906 (1993).

Where more elegant control of tryptamine production within the plant is desirable, one or both of the transgenes can be fused and thereby operationally linked to tissue-specific promoters. For example, in petunia, one or both of the transgenes can be fused to petal-specific promoters to confer resistance to infection by *Botrytis cinerea*.

Constructs according to this aspect of the invention can also comprise a selectable marker gene, expression of which by the transformed cell enables one to identify and isolate the transformed cell or cells from amongst other cells. Any plant selectable marker gene known in the art can be used in the present invention. In a preferred embodiment, the selectable marker gene is the nptII (neomycin phosphotransferase II) or hph (hygromycin phosphotransferase) gene. Cells expressing these preferred selectable marker genes are resistant to kanamycin (for nuclear nptII transformation) and hygromycin (for nuclear transformation) or glyphosate (for plastid hph transformation) and can be selected for by exposing cells subject to transformation (by, *e.g.*, biolistic delivery) to media containing the minimum level of kanamycin, hygromycin, or glyphosate that kill untransformed cells. Of course, the agent to which transformed cells are subject for selection purposes should correspond to the selectable marker gene employed in the transformation.

The sequence and structure of the constituent elements of the nucleic acid constructs according to this aspect of the invention are publicly available, and constructs according to this aspect of the invention can be made by routine, art recognized techniques. Exemplary methods are described, *e.g.*, in Example 1, *infra*.

In a second aspect, the invention provides methods for enhancing the resistance of plants to phytopathogenic fungi, bacteria, and nematodes. In a preferred embodiment, the method comprises transforming plant tissue with a construct according to the first aspect of the invention. In another embodiment, when co-transformation with both the TDC and ASα1 genes is desired, the two genes can be on separate expression vectors and co-transformed simultaneously or sequentially.

Transformation can be accomplished in either the nucleus or the plastid, as determined by the targeting regions of the nucleic acid construct. Details of plastid transformation can be found, *e.g.*, in co-pending international application PCT/US98/**** (WO 99/*****), entitled, "Improved Plastid Transformation Of Higher Plants And Production Of Transgenic Plants With Herbicide Resistance," filed July 23, 1998, and U.S. Application Serial No. 08/899,061, filed July 23, 1997.

Any of the numerous methods for transformation can be used, *e.g.*, *Agrobacterium* (for nuclear transformation), PEG treatment, electroporation, and biolistic delivery. Preferably, biolistic delivery is employed.

In a third aspect, the invention provides transgenic plant tissue that expresses the TDC gene and/or the ASα1 gene. As used herein, "plant tissue" includes a plant cell or cells, multicellular plant tissue, and whole plants. Transgenic plants according to this aspect of the invention can be made according to the second aspect of the invention using nucleic acid constructs according to the first aspect of the invention. When cells (*e.g.*, cell suspensions or calli) or plant tissue samples (*e.g.*, leaf or other plant parts) are transformed and selected, whole plants can be obtained using standard, art recognized methods of culturing and growth.

All plants are suitable for transformation with the TDC gene according to the invention. Agricultural and horticultural plants are of considerable importance, but many are susceptible to microbial infection, requiring extensive application of chemicals for disease control. A reduction in chemical applications because of increased plant resistance is always desirable for worker safety and environmental reasons.

The present invention will now be illustrated by examples that are provided solely for purposes of illustration and are not intended, nor should they be construed to be limiting in any way. Those skilled in the art will appreciate that variations and modifications of the following can be made without exceeding the scope or spirit of the invention.

EXAMPLES

Example 1

Construction of TDC- and/or AS-containing constructs

To increase tryptamine production throughout the entire plant, the TDC gene was preferably placed under the control of two very active, constitutively expressed promoters. The CaMV 35S RNA promoter from the CaMV genome is a very well-characterized promoter for expression of transgenes in both dicotyledonous and monocotyledonous plants. The enhanced version (Kay *et al.*, (1987) *Science* **236**, 1299-1302,) of the CaMV 35S RNA promoter (E35S) was preferably used. A second promoter, UBQ3, is derived from *Arabidopsis thaliana* and normally directs expression of a member of the polyubiquitin gene family. The present inventors have determined that this promoter directs high levels of reporter gene expression throughout the entire plant in transgenic petunias.

Both promoters were moved into a pUC-based vector already containing the 3′ flanking region of the nopaline synthase gene (*nos*) from *Agrobacterium tumefaciens*. This DNA sequence element possesses the recognition site for polyA addition to the transcript. Also already resident on this plasmid was a multi-cloning site region containing a number of unique restriction enzyme sites for insertion of additional DNA sequence elements. The CaMV 35S and UBQ3 promoters were moved into this plasmid (while maintaining a number of unique restriction sites between the promoter and the *nos* 3′ sequence element) to create plasmids pSAN14 and pSAN151, respectively.

The TDC gene was originally cloned from *Catharanthus roseus*, or vinca (De Luca *et al.*, (1989), *supra*. A 1.75 kbp fragment containing the full-length cDNA was cloned from a cDNA library by immunodetection methods. The TDC cDNA contains an open reading frame coding for a protein of 500 amino acids, corresponding to a molecular mass of ~56 kDa. The cDNA from plasmid pTDCS (De Luca *et al.*, 1989, *supra*) was removed by digestion with Pst I and Xho 1, the single-strand overhangs at each end removed by treatment with T4 DNA polymerase, and cloned into the Sma I sites of plasmids pSAN14 and pSAN151 to create plasmids pSAN213 and pSAN247,

respectively (Figure 1). This effectively fused the 5′ UTR region of the TDC gene to the 5′ UTR regions of the transcripts of the gene promoters. Since these constructs were to be introduced via particle bombardment, there was no direct requirement to transfer them to a T-DNA-based plant vector like pBIN19. Also, since the TDC-containing plasmids were to be co-bombarded with a second plasmid containing a plant selectable marker gene like nptII (neomycin phosphotransferase 11) or hph (hygromycin phosphotransferase), no marker gene was added to the TDC-containing plasmid.

Several gene constructs containing the ASα1 gene alone or in combination with the TDC gene were constructed. Construct pSAN368 contains the ASα1 gene driven by the UBQ10 promoter and, constructs pSAN369 and pSAN310 contained in addition to the ASα1 gene the TDC gene driven by the UBQ3 pormoter.

Example 2

Transformation of Petunia with TDC gene

The TDC transgenes were introduced into petunia by particle bombardment. Briefly, two plasmids, one containing the TDC transgene and the second containing an nptII transgene, were co-precipitated in equivalent amounts onto ~ 1 μm M-10 tungsten particles. The DNA coated particles were then bombarded into petunia leaf explants that had been placed on a nutrient agar media. After two successive bombardments, the leaf explants were allowed to recover on the nutrient media for four to five days. After recovery, the leaf explants were cut into small pieces and placed onto selective agar media containing kanamycin. Kanamycin-resistant transformed shoots were regenerated and shoots were transferred to fresh media and rooted in the absence of kanamycin.

Small leaf samples were excised from *in vitro*-maintained plantlets and cell-free extracts prepared for HPLC analysis of tryptophan and tryptamine content. In untransformed petunia plantlets that were maintained under *in vitro* conditions, tryptamine levels were approximately 4 μg per gram fresh weight (μg/gfw) of leaf tissue. In TDC-expressing petunias, transgenic lines accumulated in excess of 300 mg/gfw, approaching 100-fold over endogenous levels.

Example 3

Inhibition of fungal pathogens by tryptamine

Isolates of several phytopathogenic fungi were grown on nutrient agar media under conditions that encouraged sporulation. Conidiospore suspensions were prepared from *Fusarium*

solani, *Fusarium graminearum*, *Thielaviopsis basicola*, and *Botrytis cinerea*. For *Phytophthora parasitica* zoospores were prepared from liquid culture. For *Rhizoctonia solani* R2, a preparation of mycelial fragments (avg. length of 4 cells) substituted for the spores. In a 96-well microtiter plate, 1 x 10^3 spores (except where noted), either germinated or non-germinated, in 50 µl 0.005% Tween 20 were added to 50 µl aliquots of serially-diluted tryptamine prepared in potato dextrose broth. Microtiter plates were covered and incubated at room temperature with gentle shaking for 24-48 h. After 48 h, the lowest concentration of tryptamine which inhibited all fungal growth was designated the minimum inhibitory concentration (MIC) value. In some cases, spores were pre-incubated to allow germination to occur before exposure to tryptamine. The results are shown in Table 1.

Table 1

Tryptamine minimum inhibitory concentration (MIC) values for fungal phytopathogens

Phytopathogen	MIC[a] (mg/ml)
Phytophthora parasitica (vinca)	
non-germinated (1 x 10^2)	0.25
germinated (1.5 x 10^2)	0.25-0.50
Phytophthora parasitica (petunia)	
non-germinated	NA[b]
germinated (2 x 10^2)	0.25-0.50
Fusarium solani	
non-germinated	>1.00
germinated	0.25-0.50
Fusarium graminearum	
non-germinated	>0.50
germinated	NA
Thielaviopsis basicola	
non-germinated	0.25
germinated	0.25
Botrytis cinerea	
non-germinated	0.50
germinated	NA

Phytopathogen	MIC[a] (mg/ml)
Rhizoctonia solani R2 mycelial fragments (1×10^2)	>1.00

[a] MIC: minimum tryptamine concentration required to inhibit all fungal growth after 48 hours.
[b] not assayed

As can be observed in Table 1, non-germinated spores from the vinca isolate of *Phytophthora parasitica* were inhibited by 0.25 mg/ml tryptamine. Germinated spores from both *Phytophthora* isolates were sensitive to 0.25-0.50 mg/ml tryptamine. Similar results were observed for *Thielaviopsis basicola* (germinated and non-germinated spores). Regarding the *Fusarium* species, non-germinated spores from *F. graminearum* were somewhat sensitive to tryptamine while those from *F. solani* were considered insensitive. However, germinated spores from *F. solani* were sensitive. Also, growth from nongerminated spores of *Botrytis cinerea* were inhibited by 0.50 mg/ml tryptamine. *Rhizoctonia solani* mycelial fragments were unaffected by 1 mg/ml tryptamine. In general, nearly all the fungi exhibited some level of sensitivity to treatment with tryptamine.

Example 4

Fungicidal and Fungistatic activity of tryptamine

To determine whether tryptamine's mode of action was fungicidal or fungistatic, *Botrytis cinerea* spores were exposed to toxic levels of tryptamine for various times, then diluted to sub-lethal concentrations to permit growth. If the spores now germinated and grew, then tryptamine's effect was considered to be fungistatic. If no growth was observed, the effect was concluded to be fungicidal.

In a 96-well microliter plate, 1×10^3 *Botrytis* spores (in 50 µl 0.005% Tween 20) were treated with serial dilutions of tryptamine. The experiment was set up with three treatments. One treatment was incubated at room temperature and the wells were scored for growth after 48 h and 96 h. The second treatment was incubated at room temperature for 22 h, the wells scored for growth, then diluted 4-fold with 0.5X potato dextrose broth before continuing incubation for another 48 h and a final reading for growth. The third and last treatment was incubated at room temperature for 46 h, the wells scored for growth, then diluted 4-fold with 0.5X potato dextrose broth before continuing incubation for another 48 h and a final reading for growth.

As can be observed in Table 2, *Botrytis* spores maintained in 0.50 mg/ml tryptamine throughout the entire experiment (growth noted at 46 and 94 h) showed essentially no growth while

all growth was inhibited at 1 mg/ml. *Botrytis* spores exposed to 1 mg/ml tryptamine for 22 h showed no growth, but when diluted to 0.25 mg/ml, germinated and grew normally over the next 48 h. Similarly, spores exposed to 2 mg/ml for 22 h showed no growth, but when diluted to 0.5 mg/ml, germinated and grew, albeit relatively poorly. These results indicated that exposure to tryptamine concentrations as high as 2 mg/ml for 22 h was not toxic to *Botrytis*. Spores exposed to 0.5 mg/ml tryptamine for 46 h showed little growth, but when diluted to 0.12 mg/ml, grew normally. However, spores exposed to 1 mg/ml and 2 mg/ml tryptamine showed no growth after 46 h, and after 4-fold dilution, either grew very poorly (1 mg/ml reduced to 0.25 mg/ml) or not at all (2 mg/ml reduced to 0.5 mg/ml) over the next two days. These results suggested that tryptamine was indeed killing the *Botrytis* spores. Thus, it appears as though tryptamine can act as both a fungistatic and fungicidal compound (at least against *Botrytis)*, being fungicidal only when the concentration and exposure period are high enough and long enough, respectively.

Table 2

Tryptamine acts primarily as a fungistatic agent against Botrytis cinerea *rather than a fungicidal one*

Initial Concentration (μg/ml)	Duration (hours)	Growth	Reduced Concentration	Duration (hours)	Growth
0.00	46	+	NA	48	+
0.12	46	+	NA	48	+
0.25	46	+	NA	48	+
0.50	46	+/-	NA	48	+/-
1.00	46	-	NA	48	-
2.00	46	-	NA	48	-
0.00	22	+	0.00	48	+
0.12	22	+	0.03	48	+
0.25	22	+	0.06	48	+
0.50	22	+/-	0.12	48	+
1.00	22	-	0.25	48	+
2.00	22	-	0.50	48	+/-

Initial Concentration (μg/ml)	Duration (hours)	Growth[a]	Reduced Concentration	Duration (hours)	Growth
0.00	46	+	0.00	48	+
0.12	46	+	0.03	48	+
0.25	46	+	0.06	48	+
0.50	46	+/-	0.12	48	+
1.00	46	-	0.25	48	+/-
2.00	46	-	0.50	48	-

[a] where + represents normal growth; +/- represents dramatically reduced growth; and - represents no visible growth.
[b] not applicable

Example 5

Inhibition of phytobacteria by tryptamine

In a series of experiments similar to those described in Example 3, a collection of phytopathogenic bacteria were tested for their sensitivity to tryptamine. Cultures of *Agrobacterium tumefaciens*, *Erwinia amylovora*, *Erwinia carotovora*, *Pseudomonas cichorii*, *Pseudomonas syringae*, and *Xanthomonas campestris* were grown to saturation density. After dilution and growth for 7-8 h, cells from log-phase cultures were treated with tryptamine. Log-phase cells were grown in LB broth at 28°C (OD_{590} = 0.05 -0.15) and 50 μl of cells added to 50 μl of serial dilutions of tryptamine made in LB broth. Microtiter plates were incubated overnight at 28°C. The following day, the lowest concentration of tryptamine which inhibited all bacterial growth was recorded as the minimum inhibitory concentration (MIC) value. Table 3 shows the results.

Table 3

Tryptamine minimum inhibitory concentration (MIC) values for bacterial phytopathogens

Phytopathogen	MIC[a] (mg/ml)
Agrobacterium tumefaciens	0.50-1.00
Erwinia amylovora	>1.00
Erwinia carotovora	>1.00
Pseudomonas cichorii	0.25-0.50
Pseudomonas syringae	0.50-1.00

Phytopathogen	MIC[a] (mg/ml)
Xanthomonas campestris	0.25-0.50

[a]MIC: minimum tryptamine concentration required to inhibit all bacterial growth after 24 hrs.

P. cichorii and *X. campestris* cells exhibited greatly reduced growth in the presence of 0.25 mg/ml tryptamine, and no growth at 0.5 mg/ml. *A. tumefaciens* and *P. syringae* cells showed dramatically reduced growth at 0.5 mg/ml and were completed inhibited by 1 mg/ml. In contrast, both *Erwinia* strains grew, albeit more slowly, in the presence of 1 mg/ml tryptamine and were considered to be relatively resistant. Overall, the results in Table 3 clearly support the notion that tryptamine possesses anti-bacterial properties. Taken together with the *in vitro* anti-fungal results presented in Examples 3 and 4, tryptamine exhibits impressive broad spectrum, of antimicrobial properties.

Example 6

Tryptamine tissue distribution and tryptophan dependence in transgenic petunia

Three TDC-expressing transgene petunia lines were produced and carefully evaluated for their tryptamine levels and disease resistance characteristics. Petunia line PE-261 contains the TDC gene under control of the E35S promoter while lines PE-310 and PE-312 contain the TDC gene under control of the UBQ3 promoter. Tryptamine levels were measured in the leaf tissue of both *in vitro* and greenhouse-grown plants. Leaf tissue from *in vitro* and greenhouse plants and petal tissue from greenhouse plants (all ~10-20 mgs fresh weight) were excised and homogenized in 0.2 ml cold 50 mM Na_3PO_4, pH 7.4 buffer. Cell debris was removed by centrifugation at 10,000 x g for 5 min at 4 °C. The cleared supernatant was moved to a fresh tube and assayed for tryptamine content by HPLC using a fluorescence detector (excitation at 278 nm and emission at 360 nm). Known amounts of authentic tryptamine (Sigma) were chromatographed to construct a standard curve for the determination of tryptamine levels in the experimental samples. The results are presented in Table 4.

Table 4

Tryptamine accumulation in TDC-expressing petunias

Line	Tryptamine, µg/gfw		
	Leaf, Tissue culture	Leaf, Greenhouse	Petal, Greenhouse
V26	4	<1.4	6

Line	Tryptamine, µg/gfw*		
	Leaf, Tissue culture	Leaf, Greenhouse	Petal, Greenhouse
PE-261	310	80	310
PE-310	215	58	592
PE-312	141	42	478

* µg tryptamine per gram fresh weight of tissue

As can be seen, under tissue culture conditions, the tryptamine levels ranged from 141-310 µg/gfw. However, under greenhouse conditions, the levels decreased approximately 4-fold for all three lines to 42-80 µg/gfw. It is especially worth noting that this decrease can most likely be attributed to a reduction in the level of available tryptophan in the cell. The levels of tryptophan in the leaves of the untransformed V26 control and each of the transgenic lines dropped ~4-fold when plants were transferred to the greenhouse. In all three transgenic lines, the ratio of tryptamine to tryptophan content in the cells remained unchanged. These results strongly support the idea that tryptophan availability may be a crucial limiting factor in the production of tryptamine within the leaf.

As further evidence that tryptophan availability is rate-limiting for the *in planta* production of tryptamine in the transgenic petunias, the following experiment was devised. Stem cuttings from an untransformed V26 plant and line PE-261 were placed into tubes containing either water or 1 mg/ml tryptophan, and then incubated at room temperature for three days to permit uptake. After 3 days, leaf tissue samples were assayed for tryptophan and tryptamine levels. The results are presented in Table 5.

Table 5

Tryptamine production in TDC-expressing petunias is limited by tryptophan availability

Line	Treatment	*Tryptophan, µg/gfw*	Tryptamine, µg/gfw
V26	Water	42	2.8
	Tryptophan	18,026	3.3
PE-261	Water	11	81
PE-261	Tryptophan	2,785	2,263

As can be observed in Table 5, the V26 control samples in either water or tryptophan accumulated the same low level of tryptamine (3-4 mg/gfw), even though tryptophan levels were dramatically higher in the tryptophan-treated sample (nearly 70-fold higher). Line PE-261 in water showed the

expected levels of tryptophan and tryptamine (81 mg/gfw). However, when PE-261 was supplied with exogenous tryptophan, tryptamine levels soared to 2,263 mg/gfw, a 28-fold increase over the water treated sample. Interestingly, the level of tryptophan in the tryptophan-treated PE-261 line remained 7-fold below the V26 sample treated with tryptophan suggesting that the conversion of tryptophan to tryptamine was extremely efficient and rapid. Taken together, these results provided convincing evidence that the *in planta* production of tryptamine by TDC is limited by tryptophan availability.

Example 7

Transgenic TDC-expressing Petunia resistance to powdery mildew

Greenhouse-grown petunia lines PE-261, PE-310, PE-312 and an untransformed V26 control were inoculated on their leaf surface with a 5 µl droplet containing 10^3 powdery mildew spores. The third leaf (from the top) on each of five shoots of a single plant was inoculated. Each line was represented by four plants (for a total of 20 inoculation sites). Disease progression was monitored for approximately two weeks by recording the percentage of inoculated sites infected and measuring the diameter of the fungal colony with calipers to calculate their size (area). The results are displayed in Table 6.

Table 6

Line	Disease incidence[a]	Disease severity[b]
V26	100	100
PE-261	30	6
PE-310	36	11
PE-312	55	12

[a] percent infection of inoculated sites relative to an untransformed control assigned a value of 100
[b] relative colony size (area) compared to an untransformed control assigned a value of 100

In all three transgenic lines, disease incidence was reduced compared to inoculated, untransformed controls. The incidence of disease was reduced 2-fold for PE-312 and approximately 3-fold for lines PE-261 and PE-310. The reduction in disease incidence correlated well with the tryptamine levels in the leaf (lowest in PE-312, 42 µg/gfw, and highest in PE-261, 80 µg/gfw). Moreover, disease severity was also reduced compared to the untransformed control line. For lines PE-310 and PE-312, disease severity was reduced approximately 9-fold while disease severity for

PE-261 was reduced nearly 17-fold. Taken together, these data strongly support the argument that tryptamine-accumulating petunias show enhanced resistance to infection by powdery mildew. It is especially worth noting that reductions in both disease incidence and severity correlated extremely closely with tryptamine amounts in the leaf tissue of the three different lines. It should also be mentioned that the in *vitro* sensitivity of powdery mildew to tryptamine remains unknown since this pathogen is an obligate parasite and cannot be assayed by our usual method.

Example 8

Transgenic TDC-expressing Petunia resistance to Botrytis cinerea

We also evaluated whether the flower petals from the TDC-expressing petunia lines showed increased resistance to infection by *Botrytis cinerea*. Flower petals were measured for their tryptamine content and shown to contain between 300 and 600 μg/gfw tryptamine. The high levels of tryptamine in the petals is directly attributable to two factors, a higher concentration of available tryptophan and promoter activity. The CAMV 35S promoter is known to be extremely active throughout the entire petunia flower. Moreover, we have discovered that the UBQ3 promoter is even more active than the 35S promoter in petunia flowers, especially in a mature flower which is fully opened.

To assess *Botrytis* resistance in the petal, newly opened flowers from greenhouse plants were detached and placed into tubes containing water. A *Botrytis* spore suspension of 250 spores/ml was sprayed to the point of runoff and the inoculated flowers placed into a humidity chamber at room temperature. The rate of disease progression was then followed for 4-5 days and petals scored on a scale of 0 (uninfected) to 5 (flower collapse). Within 25 h, the untransformed control petals began to show necrotic lesions caused by *Botrytis*. These lesions rapidly expanded so that by 97 h, most flowers had totally collapsed. In contrast, the three transgenic lines showed only mild levels of infection (small, isolated lesions) after the 4-day period. At higher inoculum levels (10^3 spores/ml), the V26 control and PE-261 lines showed equal susceptibility to infection by *Botrytis*. However, lines PE-310 and PE-312 still exhibited greatly reduced rates of infection by *Botrytis*. After 97 h, PE-310 flowers still showed only a small number of isolated lesions. Disease symptoms were only slightly more advanced in PE312 flowers as the lesions were more numerous and larger.

As previously noted in the powdery mildew experiments, there was a tight correlation between tryptamine levels and the degree of *Botrytis* resistance achieved. The strongest (PE310) and

weakest (PE-261) *Botrytis*-resistant lines contained the highest (592 µg/gfw) and lowest (310 µg/gfw) amounts of tryptamine, respectively. Moreover, it now appears that the *in vitro* studies presented in Example 3 provided meaningful results as it was predicted that ~500 µg/ml tryptamine would be required to inhibit *Botrytis* (compare to the petal values of 310-592 µg/gfw). Taken together with the powdery mildew results, a very unifying and consistent picture emerges that shows that tryptamine can confer enhanced levels of disease resistance against fungal pathogens, and that the degree of resistance achieved is directly dependent upon the *in planta* tryptamine concentration.

Example 9

Sensitivity of nematodes to tryptamine

Experiments similar to those described in Example 3 were designed to test the effect of tryptamine on phytopathogenic nematodes. Larvae of the Root Knot nematode (*Meloidogyne hapla*) were exposed to various concentrations of tryptamine to test their sensitivity. A suspension of nematodes was prepared by extracting them from soil around heavily infected tomato plants. In a 24-well plate, 100-150 root-knot nematodes were added in a volume of 285 µl, next 215 µl of tryptamine solution was added to each well. The final concentrations of tryptamine were based on a 500 µl volume. Microtiter plates were covered and incubated at 25 C with gentle shaking for 48 h. At 48 h the number of mobile and immobile nematodes in each well was counted in a counting chamber using a dissecting microscope.

As can be observed in Table 7, tryptamine is very active against the root-knot nematodes. Nematodes were immobilized at 31 µg/ml, a concentration readily achieved in transgenic plants (see Example 6).

Table 7

Tryptamine conc. (mg/ml)	Number of nematodes	
	Mobile	Immobile
Experiment 1		
0	110	1
0	100	3
0.125	17	118
0.250	7	170
0.500	6	177

	Number of nematodes	
1.0	2	181
Experiment 2		
0	140	3
0	139	2
0.016	100	4
0.031	6	113
0.062	7	87
0.125	0	145

To determine if TDC-expressing plants are resistant to nematodes, leaf disks of transgenic petunia lines were inoculated with the foliar nematode *Aphelenchoides fragariae*. Briefly, leaf disks (2.1 cm diameter) were cut from surface sterilized greenhouse grown leaves of the transgenic V26 petunia lines (PE-310 and PE-312) and the non-transgenic control. Disks were placed onto wet filter paper in a multi-well dish (1 disk/well) and then inoculated with a drop containing 50 *A. fragariae* nematodes that had been raised on a sterile tobacco callus culture. Inoculated disks were incubated for eighteen days at room temperature and then the number of nematodes per well was quantified. Although, there was an increase in nematode population in each well, the two transgenic lines (PE-310, PE-312) showed only a slight increase (from 50 to 74 or 67 respectively) while the non-transgenic control increased four fold (from 50 to 194).

We have shown that two unrelated plant pathogenic nematode species are sensitive to tryptamine either by direct contact in solution (*M. hapla*) or by feeding on transgenic petunia that overexpresses TDC (*A. fragariae*). These species were selected solely on the basis of being readily available and should not represent a biased sample. We conclude that tryptamine is a generally effective nematicide. Therefore, TDC transgenic plants of any species overexpressing the TDC and/or AS gene should be resistant to nematodes, as long as the target tissue has adequate amounts of tryptamine. In the plants we have tested, tryptophan and tryptamine levels are low in the roots. Therefore, to protect roots from nematodes it may be necessary to use an AS gene to boost tryptophan and tryptamine levels in roots. In the case of root pathogenic nematodes such as *Meloidogyne* sp. and *Heterodera* sp., they induce special plant structures for feeding. These specialized feeding cells act as plant metabolic sinks. These specialized feeding cells may have

enough tryptophan for the TDC gene to produce a concentration of tryptamine that is efficacious to root feeding nematodes, even without the booster affect of an AS gene.

Example 10

Botrytis cinerea *resistance in other transgenic plant species*

Several other plant species (poinsettia cv. Angelika, geranium Designer Scarlet, lisianthus and another bedding plant) were transformed with the TDC gene. A measurable increase in tryptamine was found in transgenic poinsettia and geranium lines. Transgenic poinsettia lines demonstrated resistance to *Botrytis cinerea* in a leaf disk assay. Briefly, twelve leaf disks (8 mm in diameter) from each tissue culture-maintained transformed lines (and an untransformed control) were punched out with a cork borer and placed onto moistened Whatman 3M paper inside a sterile plastic bioassay dish. A freshly-prepared suspension of *Botrytis* spores (10^3 spores in 2.5 µl) was then pipetted onto the leaf disk surface. The humidity chamber was sealed and the leaf disks left at 20°C to permit disease development. For the next 3-14 days (timeline is species-dependent), disease progression was monitored and recorded as percentage of leaf disks infected.

Transgenic geranium lines demonstrated resistance to *Botrytis cinerea* infection when flowers were inoculated.

In at least one transgenic plant species we observed increased tryptophan and tryptamine concentration when the AS/TDC combination was used.

We claim:

1. A transgenic plant tissue having improved resistance to fungi, bacteria, nematodes or combinations thereof, comprising a plant tissue whose cells are transformed with and express a TDC transgene.

2. A transgenic plant tissue having improved resistance to fungi, bacteria, nematodes or combinations thereof, comprising a plant tissue whose cells are transformed with and express an AS transgene.

3. The transgenic plant tissue according to claim 2, wherein the AS transgene is the ASα1 transgene.

4. The transgenic plant tissue according to claim 1, wherein the plant cells are co-transformed with and express an AS transgene.

5. The transgenic plant tissue according to claim 4, wherein the AS transgene is the ASα1 transgene.

6. The transgenic plant tissue according to any one of claims 1 to 5 wherein plant tissue plastids are transformed with a TDC transgene, an AS transgene, or both.

7. The transgenic plant tissue according to claim 6, wherein the AS transgene is the ASα1 transgene.

8. A nucleic acid construct comprising a TDC gene operatively linked to the UBQ3 promoter.

9. A nucleic acid construct comprising a TDC gene operatively linked to the CaMV 35S RNA promoter.

10. A nucleic acid construct comprising a TDC gene and an AS gene.

11. The nucleic acid construct according to claim 10, wherein the AS gene is the ASα1 gene.

12. The nucleic acid construct according to claim 10, wherein the TDC gene is operatively linked to the UBQ3 promoter.

13. The nucleic acid construct according to claim 10, wherein the TDC gene is operatively linked to the CaMV 35S RNA promoter.

14. The nucleic acid construct according to claim 10, wherein the AS gene is operatively linked to the UBQ10 promoter.

15. A method of enhancing resistance to phytopathogenic bacteria, fungi, and/or nematodes in a plant tissue, the method comprising transforming the plant tissue with a nucleic acid construct according to claim 8.

16. A method of enhancing resistance to phytopathogenic bacteria, fungi, and/or nematodes in a plant tissue, the method comprising transforming the plant tissue with a nucleic acid construct according to claim 9.

17. A method of enhancing resistance to phytopathogenic bacteria, fungi, and/or nematodes in a plant tissue, the method comprising transforming the plant tissue with a nucleic acid construct according to claim 10.

18. A method of enhancing resistance to phytopathogenic bacteria, fungi, and/or nematodes in a plant tissue, the method comprising transforming the plant tissue with a nucleic acid construct according to claim 11.

19. A method of enhancing resistance to phytopathogenic bacteria, fungi, and/or nematodes in a plant tissue, the method comprising transforming the plant tissue with a nucleic acid construct according to claim 12.

20. A method of enhancing resistance to phytopathogenic bacteria, fungi, and/or nematodes in a plant tissue, the method comprising transforming the plant tissue with a nucleic acid construct according to claim 13.

21. A method of enhancing resistance to phytopathogenic bacteria, fungi, and/or nematodes in a plant tissue, the method comprising transforming the plant tissue with a nucleic acid construct according to claim 14.

Figure 1

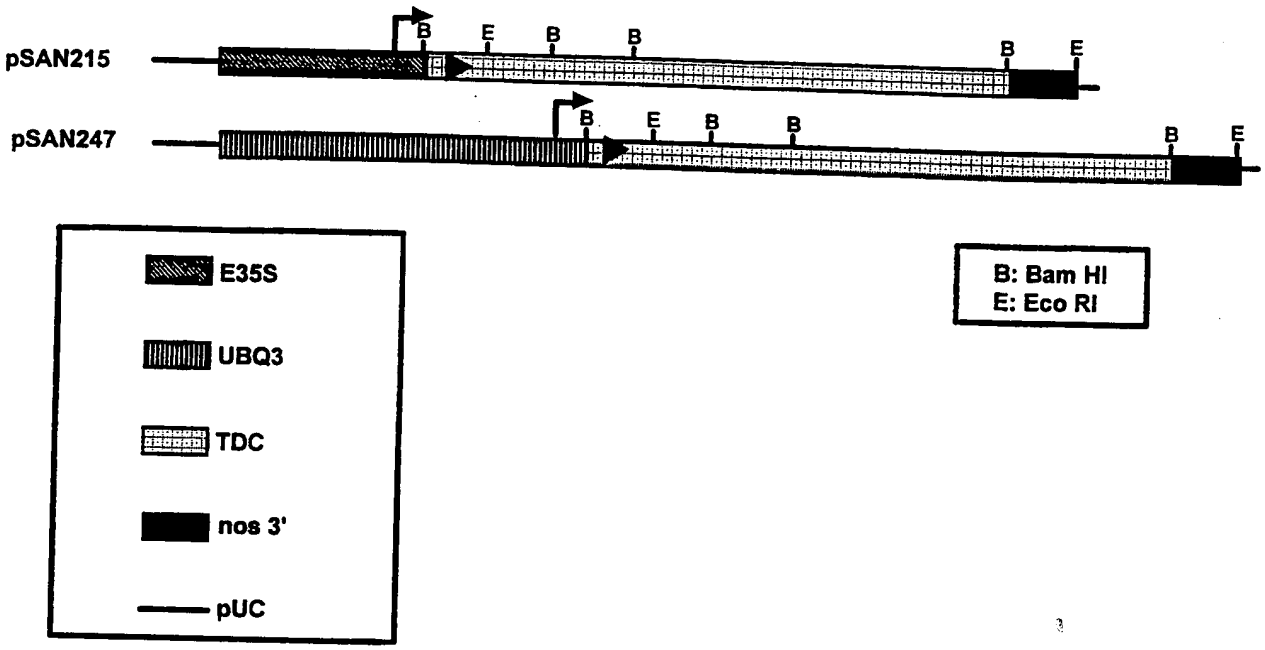
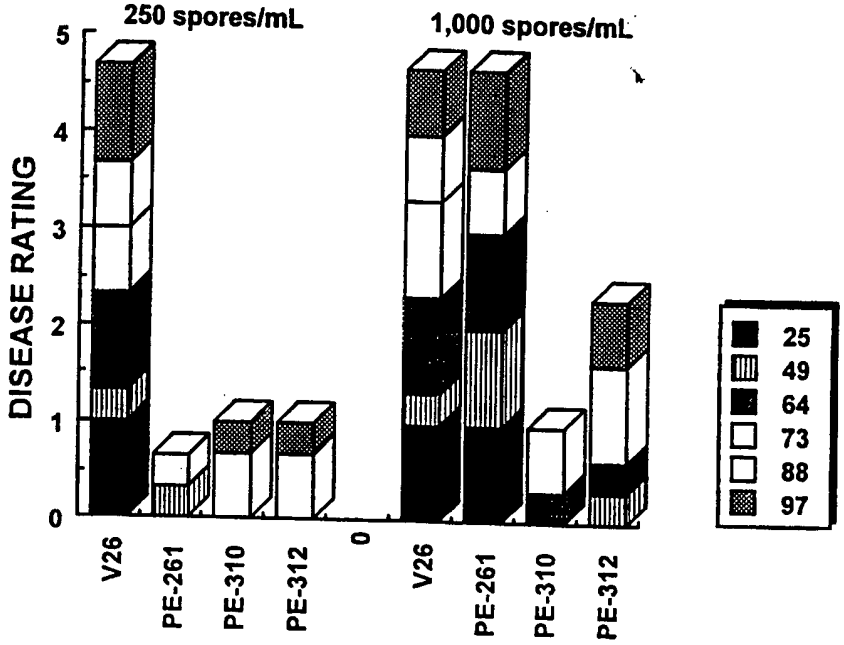


Figure 2


250 spores/mL
1,000 spores/mL
DISEASE RATING
0
1
2
3
4
5
V26
PE-261
PE-310
PE-312
0
V26
PE-261
PE-310
PE-312
25
49
64
73
88
97

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/82 C12N15/60 C12N9/88 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	WO 90 10073 A (CANADA MAJESTY IN RIGHT OF) 7 September 1990 page 3,7-15	1 16
Y	DE 41 38 209 A (SEIFERT KARLHEINZ DR ;UNGER WIBKE DR (DE)) 27 May 1993 see the whole document	1,16
X	SONGSTAD, D.D., ET AL. : "high levels of tryptamine accumulation in transgenic tobacco expressing tryptophan decarboxylase" PLANT PHYSIOLOGY, vol. 94, 1990, pages 1410-1413, XP002085254	9
Y	see the whole document	1,16

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
20 November 1998	08/12/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Holtorf, S

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INTERNATIONAL SEARCH REPORT

Interi ial Application No
PCT/US 98/16033

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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Figure 1

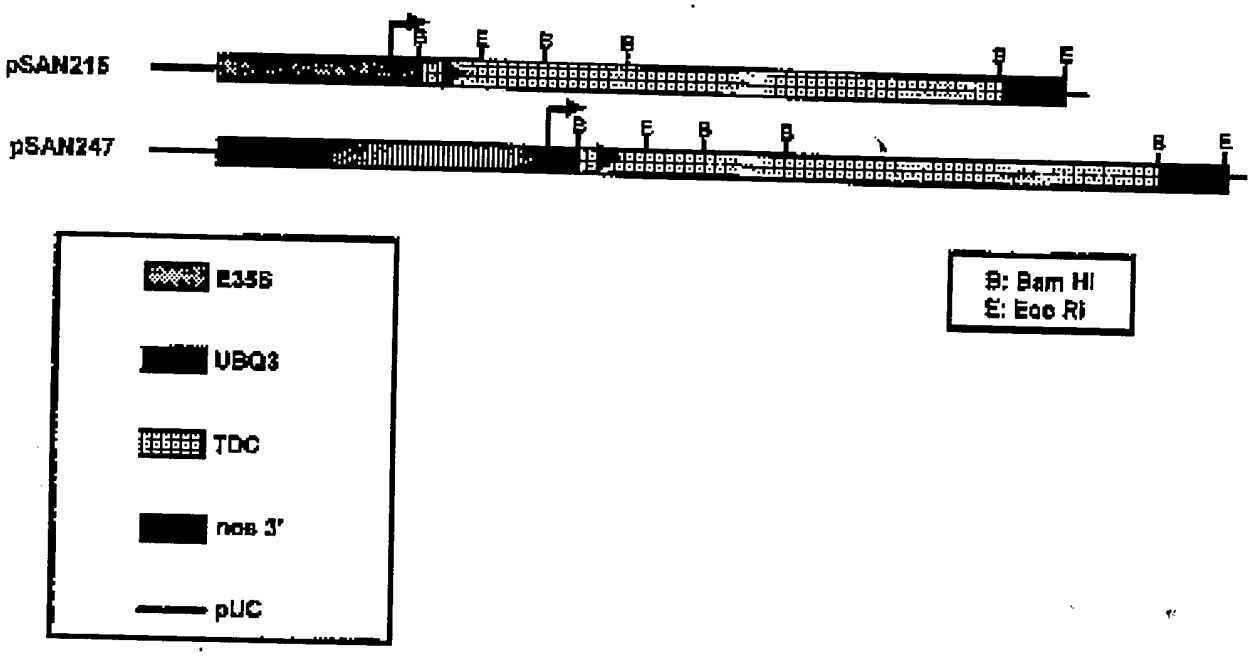

Figure 2